

**CATALYTICALLY INACTIVE TRUNCATED
GUIDE RNA COMPOSITIONS AND
RELATED METHODS FOR SUPPRESSION
OF CRISPR/CAS OFF-TARGET EDITING**

**CROSS-REFERENCE TO RELATED
APPLICATION**

[0001] This application claims the priority benefit of U.S. Provisional Application No. 62/959,710, filed Jan. 10, 2020, which is incorporated herein by reference for all purposes.

**STATEMENT OF GOVERNMENT LICENSE
RIGHTS**

[0002] This invention was made with Government support under Grant Nos. F30 CA189793 and RO1 GM109110, awarded by the National Institutes of Health and Grant No. 0954242, awarded by the National Science Foundation. The Government has certain rights in the invention.

**STATEMENT REGARDING SEQUENCE
LISTING**

[0003] The sequence listing associated with this application is provided in text format in lieu of a paper copy and is hereby incorporated by reference into the specification. The name of the text file containing the sequence listing is UWOTL173209_Sequence_final_20210108.txt. The text file is 50 KB; was created on Jan. 8, 2021; and is being submitted via EFS-Web with the filing of the specification.

BACKGROUND

[0004] The Cas9 nucleases such as *S. pyogenes* Cas9 (SpCas9) is targeted to specific sites in the genome by a single guide RNA (sgRNA) containing a 20-nucleotide target recognition sequence. The target site must also contain an NGG protospacer adjacent motif (PAM). This multipartite target recognition system is imperfect, and most sgRNAs direct significant cleavage and subsequent unwanted editing at off-target sites whose sequence is similar to the target site. Numerous approaches to reduce off-target editing have been devised yet are hampered by various limitations. For example, SpCas9 variants with improved specificity have been engineered. While useful, these high-specificity variants often decrease on-target editing, and in most cases, do not eliminate all unwanted editing. All high-specificity Cas9 variants appear to balance on- vs off-target activity via the same mechanism and, as a consequence, often fail to suppress editing at the same obstinate off-target sites.

[0005] Accordingly, despite the advances in the art of directed gene editing, a need remains for new methods for off-target suppression, particularly methods that preserve on-target editing, and which can be combined with high-specificity nucleases variants, while requiring minimal expenditure of time, effort, and resources. The present disclosure addresses these and related needs.

SUMMARY

[0006] This summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This summary is not intended to identify key features of the claimed subject

matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

[0007] In one aspect, the disclosure provides a method of inhibiting off-target cleavage of a DNA molecule by a first guide RNA-endonuclease complex, wherein the first guide RNA-endonuclease complex comprises a first guide RNA comprising a nucleotide target recognition sequence complementary to a first target sequence. The method comprises contacting the DNA molecule with a second guide RNA-endonuclease complex, wherein second guide RNA-endonuclease complex comprises a second guide RNA comprising a nucleotide target recognition sequence with 16 or fewer nucleotides and is complementary to a second target sequence in the DNA molecule. The second target sequence is different from the first target sequence but the second target sequence is capable of cleavage at a measurable rate by the first guide RNA-endonuclease complex.

[0008] In some embodiments, the method further comprises contacting the DNA molecule with the first guide RNA-endonuclease complex. In some embodiments, the second guide RNA-endonuclease complex is contacted to the DNA molecule prior to or simultaneously with the first guide RNA-endonuclease complex. In some embodiments, the first guide RNA-endonuclease complex and the second guide RNA-endonuclease complex are contacted to the DNA molecule at a ratio of about 20:1 to about 1:20. In some embodiments, the second target sequence differs from the first target sequence by 0-10 nucleotide mismatches.

[0009] In some embodiments, the first guide RNA-endonuclease complex comprises a first endonuclease and the second guide RNA-endonuclease complex comprises a second endonuclease, wherein the first endonuclease and the second endonuclease are clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system proteins.

[0010] In some embodiments, the first endonuclease and the second endonuclease are independently selected from Cas12a, Cas9, or high-fidelity variants of Cas9 such as eSpCas9, SpCas9-HF1, HypaCas9, as well as xCas9, SpCas9-NG, and the like. In some embodiments, the first endonuclease is the same type of endonuclease as the second endonuclease. In some embodiments, the first endonuclease or the second endonuclease is derived from *Streptococcus*, e.g., *Streptococcus pyogenes*, *Staphylococcus*, e.g., *Staphylococcus aureus*, *Neisseria*, e.g., *Neisseria meningitidis*, *Acidaminococcus* species, or *Lachnospiraceae* species.

[0011] In some embodiments, contacting the DNA molecule with the second guide RNA-endonuclease complex reduces cleavage of the second target sequence by the first guide RNA-endonuclease complex by at least 10% compared to similar reaction conditions but wherein no second guide RNA-endonuclease complex is present. In some embodiments, the nucleotide target recognition sequence of the second guide RNA-endonuclease complex comprises between 10 and 16 nucleotides inclusive that are complementary to the second target sequence.

[0012] In some embodiments, the method is multiplexed with one or more additional guide RNA-endonuclease complexes, wherein each of the one or more additional complexes comprises a different nucleotide target recognition sequence with 16 or fewer nucleotides and is complementary to one or more additional target sites in the DNA molecule or a plurality of DNA molecules in a same reaction environment, wherein the one or more additional target